

Experimental study of anti-tumor effects of polysaccharides from *Angelica sinensis*

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Abstract

AIM: To investigate the *in vivo* anti-tumor effects of total polysaccharide (AP-0) isolated from *Angelica sinensis* (Oliv.) Diels (Danggui) on mice and the *in vitro* inhibitory effects of AP-0 and its sub-constituents (AP-1, AP-2 and AP-3) on invasion and metastasis of human hepatocellular carcinoma.

METHODS: Three kinds of murine tumor models *in vivo*, sarcoma 180 (S180), leukemia L1210 and Ehrlich ascitic cancer (EAC) were employed to investigate the anti-tumor effects of AP-0. For each kind of tumor model, three experimental groups were respectively given AP-0 at doses of 30, 100 and 300 mg/kg by *ip* once a day for 10 days. Positive control groups were respectively given Cy at a dose of 30 mg/kg for S180 and leukemia L1210, and 5-FU at a dose of 20 mg/kg for EAC. On d 11, mice bearing S180 were sacrificed and the masses of tumors, spleens and thymus were weighed. The average living days of mice bearing EAC and of mice bearing L1210 were observed, and the rates of life prolongation of each treatment were calculated, respectively. The inhibitory effects of APs on hepatoma invasion and metastasis *in vitro* were investigated by employing human hepatocellular carcinoma cell line (HHCC) with the Matrigel invasion chamber, adhesion to extracellular matrix and chemotactic migration tests, respectively.

RESULTS: AP-0 had no obviously inhibitory effect on the growth of S180, but it could significantly decrease the thymus weights of the mice bearing S180. AP-0 could significantly reduce the production of ascitic liquids and prolong the life of mice bearing EAC. AP-0 could also increase the survival time of mice bearing L1210. AP-0 and AP-2 had significantly inhibitory effects on the invasion of HHCC into the Matrigel

reconstituted basement membrane with the inhibitory rates of 56.4 % and 68.3 %, respectively. AP-0, AP-1, AP-2 and AP-3 could influence the adhesion of HHCC to extracellular matrix proteins (Matrigel and fibronectin) at different degrees, among them only AP-3 had significant blocking effect on the adhesion of HHCC to fibronectin with an inhibitory rate of 30.3 %. AP-0, AP-1 and AP-3 could partially inhibit the chemotactic migration abilities of HHCC.

CONCLUSION: The experimental findings suggest that total polysaccharide of *Angelica sinensis* (Oliv.) Diels (Chinese Danggui) possesses anti-tumor effects on experimental tumor models *in vivo* and inhibitory effects on invasion and metastasis of hepatocellular carcinoma cells *in vitro*.

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INTRODUCTION

The Chinese herbal medicine Danggui, root of *Angelica sinensis* (Oliv.) is widely used in traditional Chinese medical therapy of various diseases as well as a healthful food tonic and spice for thousands of years. Being called the "female ginseng", it is excellent as an all purpose women's herb^[1]. Danggui can be used for anemia due to chronic renal failure (CRF)^[2] and can enhance hematopoiesis by stimulating macrophages, fibroblasts, lymphocytes in hematopoietic inductive microenvironment and muscle tissue to secrete hematopoietic growth factors. Gastrointestinal protective effects^[3,4] and the mechanism^[5,6] of polysaccharides from *Angelica sinensis* in rats have been reported. Effects of *Angelica* polysaccharides on blood coagulation and platelet aggregation^[7] and the protective effect of the polysaccharides-enriched fraction from *Angelica sinensis* on hepatic injury^[8] were also studied. A low molecular weight polysaccharide from *Angelica sinensis* (Oliv.) Diels showed strong anti-tumor activity on Ehrlich ascitic cancer bearing mice and also exhibited immunostimulating activities, both *in vitro* and *in vivo*^[9] (Choy *et al. Am J Chin Med*, 1994; 22:137). Polysaccharides from three kinds of edible fungi showed inhibitory activities on the proliferation of human hepatoma SMMC-7721 cells and mouse implanted S180 tumor^[10]. Apoptosis of hepatoma cells SMMC-7721 could be induced by polysaccharides from *Ginkgo biloba* seeds^[11]. In China, hepatocellular carcinoma with high invasiveness and recurrence has ranked second of cancer mortality since 1990s. Anti-invasion and metastasis is currently one of the major targets of hepatoma studies^[12,13]. Some polysaccharides involve in the process of tumor invasion and progression, such as heparin, heparan sulfate and hyaluronan^[14-16]. The anti-metastasis effects and their pharmacological mechanisms of modified citrus pectin^[17] and protein-bound polysaccharide (PSK) have been studied recently^[18-20]. However, the anti-tumor effects on other kinds of tumors and the anti-invasion

and metastasis effects of polysaccharides from *Angelica sinensis* (Oliv.) Diels have not been studied. In the present study, the anti-tumor effects of total polysaccharide (AP-0) on mice bearing transplanted sarcoma 180, leukemia L1210 and Ehrlich ascitic cancer (EAC) were investigated. The *in vitro* inhibitory effects of AP-0 and its sub-constituents, AP-1, AP-2 and AP-3 on the abilities of invasion and metastasis of human hepatocellular carcinoma were also tested by employing HHCC cell line.

MATERIALS AND METHODS

Animals and cell lines

Male BALB/c mice, weighing 18-22 g, were used in S180 and EAC experiments, and male DBA/2 mice, weighing 18-22 g, were used in L1210 experiment. All the mice were raised in a clear room with controlled temperature and humidity. Animals were free to drink tap water. Human hepatocellular carcinoma cell line (HHCC) was purchased from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. Human embryo dermal fibroblast cell line (Fb) was kindly presented by Dr. JT Han (Department of Plastic Surgery, Fourth Military Medical University).

Polysaccharides from Angelica sinensis

Fresh roots of *Angelica sinensis* (Oliv.) were picked in autumn from Minxian County, Gansu Province, China. Total polysaccharide (AP-0) was isolated by ethanol precipitation after boiling water extraction. The sub-constituents, AP-1, AP-2 and AP-3 from AP-0 were obtained by multi-precipitation treated with CTAB, H₃BO₃, NaOH, acetic acid and ethanol according to Yamada *et al.*^[21]. The physical-chemical characteristics of APs were analyzed and determined (Shang P, *et al. Disi Junyi Daxue Xuebao*, 2001;22:1311; *Chin Pharmaceutical J*, 2000;35:332). The contents of total carbohydrate, uronic acids and proteins in AP-0, AP-1, AP-2 and AP-3 were 97.0 %, 21.0 % and 3.0 %; 97.0 %, 17.2 % and 3.0 %; 83.0 %, 25.7 % and 17 %; 97.0 %, 8.6 % and 3 %, respectively. All of APs' solution was dispensed with sterile *N S* in the laminar flow bench.

Mice transplanted tumor models and Angelica polysaccharide (AP-0) treatments

Mice model of S180 was established by inoculating 5×10⁶ S180 cells into left armpit of each mouse. From the next day following tumor cells inoculation (d 1) to the 10th day (d 10), mice in 3 experimental groups were administrated *ip* AP-0 at doses of 30, 100 and 300 mg/kg, once daily, respectively. Positive and negative control groups were given Cy at a dose of 30 mg/kg and *N S* of 20 mg/kg, once daily, respectively. On d 11, the mice were sacrificed, and the weights of tumor, spleen and thymus were recorded. Mice model of EAC was made by injecting *ip* 2.5×10⁶ EAC cells into each mouse. From the second day of tumor cells inoculation (d 1) to the 10th day (d 10), mice in 3 experimental groups were given AP-0 *ip* at doses of 30, 100 and 300 mg/kg, once daily, respectively. Positive and negative control groups were given 5-FU at a dose of 20 mg/kg and *N.S.* of 20 mg/kg, respectively. The average survival time of less than 60 days each group was observed and the effects of AP-0 on prolongation were calculated. Mice model of L1210 was made by injecting *ip* 1×10⁵ L₁₂₁₀ cells into each mouse. From the next day of tumor cells inoculation (d 1) to the 10th day (d 10), mice in 3 experimental groups were given AP-0 *ip* at doses of 30, 100 and 300 mg/kg, once daily, respectively. Positive and negative control groups were given Cy at a dose of 30 mg/kg and *N S* of 20 mg/kg. The average survival time of less than 30 days of each group was observed and the rates of life prolongation were calculated.

Anti-invasion tests of angelica polysaccharides in vitro

Experimental methods employed in this part were based on previous report^[22] with a slight modification.

AP-0-AP-3's inhibitory effects on HHCC invasion into reconstituted basement membrane Each inner membrane of Millicell chamber (Millipore, USA) was paved with 40 μL Matrigel (BD Bioscience, USA) and dried in the laminar bench at room temperature. The Matrigel-Millicells of co-cultured HHCC and Fb, with each Millicell containing 150 μL (1.5×10⁵) cells cultured in DMEM supplemented with 1 mL/L bovine calf serum (BCS), were put into 24-well microplates (Nunc, Sweden) containing 10 μg fibronectin (FN, GIBCO, USA) as a chemotactic agent in 200 μL DMEM (GIBCO, U S A.) supplemented with 1 mL/L BCS. AP-0, AP-1, AP-2 and AP-3 at a dose of 50 μL (7.0 mg/ml) were added into the Millicell. After the microplates and Millicells were incubated for 20 hours at 37 °C, 50 mL/L CO₂ (Heraeus, Germany), each upper surface of membrane was scrubbed 3 times with a cotton swab. All of the membranes were fixed with 950 mL/L ethanol and then taken off for HE staining. HHCC cells infiltrated into the membrane were counted under a high power microscope. All experiments were performed in triplicate.

Inhibition of AP-0-AP-3 on HHCC adhesion to extracellular matrix proteins Each well of the 96-well microplates was coated with FN and Matrigel, 2 μg for each one, respectively. The coated wells were allowed to dry, then 20 μL 10 g/L BSA was added into each well and incubated for 1 hour. The wells were washed with PBS. Each well containing 200 μL (2×10⁵) HHCC and 50 μL (7.0 g/L) APs was incubated for 2 hours at 37 °C, 50 mL/L CO₂. After each well was washed 3 times with 200 μL PBS, 40 μg MTT was added into each one and incubated for 4 hours at 37 °C, 50 mL/L CO₂. Then the liquid was removed, and 200 μL DMSO was added into each well. The absorbance of each well was detected by a microplate Reader (Bio-Rad 450, USA) at 490 nm. Triplicate determinations were performed at each data point.

Influences of AP-0-AP-3 on HHCC chemotactic migration The outer-surface of each Millicell was paved with 10 μg FN as a chemotactic agent. 150 μL (1.5×10⁵) HHCC cells cultured in DMEM supplemented with 1 mL/L BCS, was added into each Millicell. Then the Millicells were cultured in 24-well microplates containing 200 μL DMEM supplemented with 1 mL/L BCS in each well. AP-0, AP-1, AP-2 and AP-3 at doses of 50 μL (7.0 g/L) were added into the Millicells respectively. After the microplates containing Millicells were incubated for 20 hours at 37 °C, 50 mL/L CO₂, each upper surface of the membrane was scrubbed 3 times with a cotton swab. Then the membranes were fixed with 950 mL/L ethanol and taken off for HE staining. The HHCC cells infiltrated into the membrane were counted under a high power microscope. All experiments were performed in triplicate.

Statistical analysis

Differences of weights, cell counts and absorbance between the experimental and control groups were examined by using ANOVA (analysis of variance). Differences of life prolongation rates were examined by Cox proportional hazards regression.

RESULTS

Effects of Angelica polysaccharide (AP-0) on mice transplanted tumors

Influences of AP-0 on immunological organs of mice bearing S180 Although the differences of tumor's weights between the three AP-0 groups and *N.S.* control group were not significant, thymus weights of mice receiving AP-0 were significantly lighter than those of mice treated by *N.S.* Whereas,

thymus weights of mice receiving AP-0 were significantly heavier than those of mice treated by Cy (Table 1). Thymus weights of mice receiving doses of AP-0 at 100 mg/kg ($P<0.05$), 30 mg/kg and 300 mg/kg ($P<0.01$) were lighter than those of NS group. The thymus weights of mice receiving doses of dAP-0 at 30 mg/kg ($P<0.05$), 100 mg/kg and 300 mg/kg ($P<0.01$) were heavier than those of Cy group.

Table 1 Masses of body, tumor, thymus and spleen of mice bearing S180 10 days after being treated with AP-0, Cy and NS ($\bar{x}\pm s$)

Group	Mice (n) (d10/d0)	m (body) (g)	m (tumor) (g)	m (thymus) (mg)	m (spleen) (g)
NS	10/10	25±3	2.6±0.8 ^d	69±17	0.25±0.08 ^d
Cy	9/9	19±3 ^b	1.2±0.4	16±10 ^b	0.06±0.02
AP-0					
30 mg/kg	9/10	22±5	2.8±1.0 ^d	35±19 ^{bd}	0.22±0.13 ^d
100 mg/kg	8/10	22±3	2.7±0.5 ^d	46±16 ^{ad}	0.20±0.08 ^d
300 mg/kg	7/10	22±2	3.0±1.3 ^d	44±14 ^{bd}	0.34±0.14 ^d

^a $P<0.05$, ^b $P<0.01$ vs NS, ^d $P<0.01$ vs Cy.

Effects of AP-0 on mice bearing EAC The average weight of mice in each group receiving AP-0 treatment was far significantly lighter than that of NS group, suggesting that the amounts of ascitic fluid produced in the mice of experimental groups were less than those of NS groups (Table 2). The survival time of each AP-0 treated group was longer than that of NS group within 60 day observation. However, significant difference was found only between the AP-0 300 mg/kg treated group and NS group (Table 2).

Table 2 Survival and body masses of mice bearing EAC after being treated with AP-0

Group	n	Survival time (days)	Life prolongation rate (%)	m (body)/(g) ($\bar{x}\pm s$)	
				d 0	d 10
NS	10	14.1	0.0	19.9±0.9	42.2±1.9
5-FU	10	60.0	325.0	20.2±0.9	24.7±3.6 ^b
AP-0					
30 mg/kg	10	26.5	89.7	19.8±1.6	36.5±5.2 ^b
100 mg/kg	10	18.4	30.5	19.4±1.2	35.8±3.0 ^b
300 mg/kg	10	36.0	155.3 ^a	19.3±1.0	31.3±3.8 ^b

^a $P<0.05$ vs NS, ^b $P<0.001$ vs NS.

Effects of AP-0 on mice bearing L1210 The survival time of mice receiving AP-0 at doses of 30 mg/kg and 100 mg/kg was longer than that of NS group within 30 day observation. Only 30 mg/kg group had a significant difference compared with N.S. group (Table 3). The effect was not dose-dependent.

Table 3 Survival of mice with L1210 after being treated with AP-0 ($\bar{x}\pm s$)

Group	n	Survival time (days)	Life prolongation rate (%)
NS	0	15.0	0.0
Cy	5	30.0	200.0
AP-0			
30 mg/kg	1	25.0	66.7 ^a
100 mg/kg	0	23.5	56.7
300 mg/kg	0	15.0	0.0

^a $P<0.05$ vs NS.

Effects of *Angelica* polysaccharides on invasion and metastasis of hepatocellular carcinoma in vitro

Inhibition of AP-0-AP-3 on HHCC infiltrating into reconstituted basement membrane AP-0, AP-1, AP-2 and AP-3 could inhibit HHCC cells to infiltrate into the reconstituted basement membrane with inhibitory rates of 56.4 %, 38.6 %, 68.3 % and 26.7 %, respectively. Among them, AP-0 and AP-2 had significant inhibitory effects ($P<0.05$) compared with the control (Table 4).

Table 4 Inhibition of *Angelica* polysaccharides on infiltration and migration of HHCC

Group	Infiltration		Migration	
	Infiltrated cells ($\bar{x}\pm s$)	Inhibitory rate (%)	Migrated cells ($\bar{x}\pm s$)	Inhibitory rate (%)
Control	20.2±18.5	0.0	23.6±18.7	0.0
AP-0	8.8±9.2 ^a	56.4	29.5±17.8	\
AP-1	12.4±9.5	38.6	14.4±4.8	39.0
AP-2	6.4±2.9 ^a	68.3	17.0±7.9	28.0
AP-3	14.8±14.5	26.7	16.3±9.0	30.9

^a $P<0.05$ vs Control.

Influences of AP-0-AP-3 on HHCC adhesion to extracellular matrix proteins AP-0, AP-1, AP-2 and AP-3 could inhibit HHCC cells adhesion to Matrigel and FN by different degrees. However, only AP-3 could significantly inhibit adhesion of HHCC cells to FN (Table 5).

Table 5 Influence of *Angelica* polysaccharides on adhesion of HHCC to Matrigel and fibronectin

Group	Matrigel		Fibronectin	
	A 490 nm ($\bar{x}\pm s$)	Inhibitory rate (%)	A 490nm ($\bar{x}\pm s$)	Inhibitory rate (%)
Control	0.131±0.029	0.0	0.099±0.023	0.0
AP-0	0.114±0.006	12.9	0.098±0.017	1.0
AP-1	0.118±0.037	9.9	0.082±0.004	17.2
AP-2	0.105±0.018	19.8	0.073±0.014	26.0
AP-3	0.098±0.020	25.2	0.069±0.008 ^a	30.3

^a $P<0.05$ vs Control.

Influences of AP-0-AP-3 on HHCC chemotactic migration abilities AP-0 had no inhibitory effect on the migration of HHCC cells, but its sub-constituents AP-1, AP-2 and AP-3 had weak inhibitory effects on the migration of HHCC cells into membranes, with no statistical significance compared with N.S. group ($P<0.05$, Table 4).

DISCUSSION

Polysaccharides from various kinds of herbal medicines or plants have extensive pharmacological activities. Since Letinan, a polysaccharide from *Lentinus edodes* (Berk.) Sing (Chihara, *et al. Nature*. 1969;22:687-688) was discovered to inhibit mouse sarcoma 180, a lot of plant polysaccharides have been investigated into their anti-tumor effects and the related mechanisms (Chihara, *et al. Nature*. 1970;225:943-944; Maeda Y, *et al. Nature*. 1971;229:634). In the present study, we examined systematically the anti-tumor effects of polysaccharides from Chinese Danggui (*Angelica sinensis*) in mice according to the criteria of anti-tumor drug evaluation. AP-0 might have no direct inhibition on murine solid tumor S180, but it could significantly decrease the weights of thymus

of mice bearing S180. Gu *et al* (*Bull Acad Mil Med Sci*.1986;10:401) reported that two types of polysaccharides from *Angelica* could not only decrease the weights of thymus, but also decrease the amounts of peripheral T and B lymphocytes of normal and tumor-bearing mice. Maeda believed that the tumor-inhibiting effect of Lentinan was mediated by T lymphocytes. Our results of the S180 experiments suggested that AP-0 had significant influences on thymus of mice, but the effects were not strong enough to inhibit the growth of tumor. By using body-mass as an indicator of the amount of ascitic fluid produced in EAC-mice, we discovered that AP-0 inhibited the production of ascitic fluid and prolonged the survival of the mice. Kumazawa, *et al* (*Immunology*. 1982; 47:75) previously found that AIP, which was an immunostimulating polysaccharide separated from hot water extract of *Angelica acutiloba* Kitagawa (Yamato Tohki), prolonged the survival of mice bearing EAC. Likewise, AP-0 inhibited the production of ascitic fluid and prolonged the life of mice bearing L1210. The results of *in vivo* experiments suggested that AP-0 had obvious anti-tumor effects on murine ascitic tumors (AEC and L1210), and also affected the immune organ (thymus) of mice bearing S180. Immunostimulatory activities found in the polysaccharides from *Panax ginseng*, *Spirulina platensis*, *Aphanizomenon flos-aquae* and *Chlorella pyrenoidosa* also suggested that immunomediated effects might be the main mechanism of polysaccharides' anti-tumor actions^[23,24]. Polysaccharides with potent anti-tumor effects have been used clinically for cancer immunotherapy combined with radiotherapy and chemotherapy^[25-27].

Invasion and metastasis are essential characteristics of malignant tumors. Tumor invasion and metastasis are a multi-step process associated with multiple-factors, such as adhesion and migration of tumor cells, degradation of extracellular matrix (ECM), and angiogenesis^[28]. Molecules existing in ECM and receptors or ligands existing on the surfaces of tumor cells played critical roles in invasion and metastasis^[29,30]. Polysaccharides, such as proteoglycans and glycosaminoglycans are the main constituents of ECM. Liu *et al*^[15] demonstrated that heparan sulfate glycosaminoglycan coat presenting on tumor cells contained bioactive sequences that impinged on tumor-cell growth and metastasis. Hyaluronan, an extracellular polysaccharide that has been implicated in tumor invasion, was one of the major constituents of stromal myxoid changes^[16]. As to the polysaccharides from herbal medicines or plants, their anti-metastatic effects have been reported. Modified citrus pectin (MCP), a nondigestible, water-soluble polysaccharide fiber derived from citrus fruit, given orally, inhibited carbohydrate-mediated tumor growth, angiogenesis, and metastasis *in vivo*^[17]. Protein-bound polysaccharide PSK inhibited tumor invasiveness by down-regulation of TGF-beta1, uPA, MMP-2 and MMP-9 expressed in two human tumor cell lines, pancreatic cancer cell line (NOR-P1) and gastric cancer cell line (MK-1P3)^[18]. Enhancement of HLA class-I expression on tumor cells after PSK treatment might be one of the mechanisms responsible for the induction of anti-tumor immunity by PSK^[19].

The present anti-metastasis study employed human hepatocellular carcinoma cell (HHCC) co-cultured with fibroblast, a kind of important stromal cell, to test the inhibitory effects of AP-0, AP-1, AP-2 and AP-3 on metastasis *in vitro*. The results of our experiments showed that AP-0 and its sub-constituents could inhibit the infiltration, adhesion and migration of HHCC by different degrees. By analyzing the biological-effect and chemical-composition, we found that there might be some relationship between the anti-invasion activities and the uronic acids containing APs. AP-0 and AP-3 had the strongest anti-degradation effects on basement membrane, and also possessed the highest amounts of uronic

acids of 210 g/kg and 257 g/kg among the 4 polysaccharides from *Angelica*. On the other hand, AP-3 with the lowest content of uronic acids had the most effective anti-adhesion action on HHCC cells to FN among the 4 polysaccharides. The polysaccharides rich in anion groups, such as heparin and heparan sulfate, are closely related to the action between cells and ECM. Based on this, it is proposed that AP rich in uronic acids act as analogues of natural polysaccharides in ECM, and exhibit their anti-metastasis effects.

The findings in the present study suggest that polysaccharides from *Angelica* not only have anti-tumor effects on murine tumors of S180, EAC and L1210 *in vivo*, but also can inhibit invasion of HHCC *in vitro*.

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